Effect of Benzalkonium Chloride on Biofilm of Bacteria Causing Nosocomial Infections

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ABSTRACT

Background and Objective: Biofilms are community of bacteria that attach to inanimate surfaces or living tissues via production of extracellular polymers and exopolysaccharide matrix. Microbial biofilms on various surfaces of the hospital environment are considered as a reservoir of infection spread. The present study aimed to evaluate the disinfecting effect of benzalkonium chloride on some bacterial isolates causing nosocomial infections.

Methods: First, 13 isolates from four bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter* and *Enterobacter* were obtained from Microbiology Laboratory of Al-Zahra Hospital in Isfahan, Iran. The samples were transferred to Microbiology Laboratory of Faculty of Veterinary Medicine of Shahrekord University for testing. Evaluation of biofilm formation and determination of minimum inhibitory concentration (MIC) of the disinfectant and effect of the disinfectant on planktonic growth and biofilm formation were performed.

Results: All bacterial isolates (52 cases) produced biofilm. Mean MIC of benzalkonium chloride for *P. aeruginosa*, *S. aureus*, *Enterobacter* and *Acinetobacter* was 0.14, 0.2, 0.18, 0.17 g/ml, respectively. Planktonic growth of all four bacteria was inhibited at concentrations of 2MIC, MIC and 1/2MIC. Biofilm was not produced in MIC and 2MIC concentrations, and biofilm formation capability increased by reducing the concentration of benzalkonium chloride.

Conclusion: The results show that the use of appropriate concentration of benzalkonium chloride can prevent the growth of different bacterial species, but sub-MIC dose of this disinfectant may stimulate biofilm formation.

Keywords: Biofilm, Benzalkonium Chloride, *Pseudomonas Aeruginosa*, *Staphylococcus Aureus*, *Enterobacter*, *Acinetobacter*.

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INTRODUCTION

For more than a century, nosocomial infections have been known as a critical issue affecting the quality of healthcare and a major source of medical complications. Bacteria and especially those from the Enterobacteriaceae family (such as *Escherichia coli*) are the most common causes of nosocomial infections (1). *Staphylococcus aureus* is part of skin normal flora that can cause illness under favorable conditions. It is also the second leading cause of nosocomial infections (2). *Pseudomonas aeruginosa* is another important bacterium in nosocomial infections that multiplies easily in water and wet locations in hospitals. It is considered a risk factor for burn patients and immunocompromised patients including those with malignancies (3). Biofilm is a mass of bacteria attached on a solid surface, surrounded by a sticky substance called exopolysaccharide. Most bacteria that cause nosocomial infections form biofilm on surface of equipment, medical supplies and even hospital floor. Biofilm formation protects bacteria from antimicrobial agents and increases their resistance to disinfectants and antibiotics (4). Several studies have been conducted on controlling bacterial biofilm using disinfectants. This study used benzalkonium for inhibition of biofilm formation. Benzalkonium, known as alkyl dimethyl benzyl ammonium chloride, has a cationic surface and is dependent to the quaternary ammonium group. This compound is an antiseptic, disinfectant and biocide that is effective against several bacteria, viruses and fungi except for bacterial spores (5). Benzalkonium has a wide range of applications in medicine and veterinary medicine. It is used as an active ingredient in commercial antiseptics such as Dettol and Lysol. In the medical sector, it is used as an antiseptic solution for surgical instruments and supplies, hospital and skin disinfection, and the treatment of herpes simplex infection and herpetic lesions (6). Benzalkonium chloride causes detachment of the cellular lipid bilayer membrane by impairing cellular interactions. As a result, permeability of the cell is disrupted, causing cell contents to be released. Based on the concentration used, this compound could have bacteriostatic or bactericidal effects (7). The main aim of this study was to determine the minimum inhibitory concentration (MIC) of benzalkonium chloride against biofilms formed by *S. aureus, P. aeruginosa, Acinetobacter* and *Enterobacter* isolates from nosocomial infections.

MATERIAL AND METHODS

Bacterial samples suspected of *P. aeruginosa, S. aureus, Acinetobacter* and *Enterobacter* were isolated from nosocomial infections at Al-Zahra hospital. Tests for verification and detection of species were performed according to methods available in diagnostic bacteriology laboratory textbook in the chloride (with 10% initial concentration) for each of the four bacteria tested, 50 µl of the disinfectant solution was mixed with 50 µl of TSB medium in the first well of the microplate. Then, 1:2 serial dilutions were made in the remaining 11 wells. Next, 0.1 ml of bacterial suspension equal to half McFarland was mixed with 9.9 ml sterile TSB. Then, 50 µl of this suspension was added to each of the 12 wells. After incubation of microplates at 37 °C for 24 hours, MIC was determined as the lowest concentration of the disinfectant that prevented visible growth of bacteria.

To evaluate the impact of benzalkonium chloride on the planktonic growth of bacteria, a bacterial suspension with concentration equal to half McFarland was prepared using the 24-hour cultures of the isolates. Then, 50 µl of the bacterial suspension and 50 µl of different concentrations of benzalkonium chloride (1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC and 2 MIC) were mixed in each well of the microplate. After 24 hours of incubation at 37 °C, the planktonic growth of bacteria at 630 nm was measured using an ELISA microplate reader. The effect of benzalkonium chloride on biofilm formation by bacteria was assessed according to Tendolkar et al. method. The aforementioned concentrations of benzalkonium chloride were added to the wells before incubation, crystal violet discarded from the biofilm of isolates was assessed based on the OD. Finally, statistical analysis was done using SPSS software (version 20) and graphs were plotted using Microsoft Excel. Comparisons of mean properties were analyzed using Duncan's multiple range test at 5% probability.

RESULTS

This study was performed on two species of *P. aeruginosa* and *S. aureus*, and two genera of *Acinetobacter* and *Enterobacter*. In order to evaluate biofilm formation, first, the OD of the sample and the negative control (ODc) was calculated. According to the difference in the OD and ODc values, results were categorized in four groups of non-biofilm forming (OD ≤ ODc), weak biofilm forming (ODc <OD ≤ 2 ODc), moderate biofilm forming (2 OD <OD <4 ODc) and strong biofilm forming (OD> 4ODc) bacteria (Table 1). Of 13 *S. aureus* isolates, four (30.7%) were weak biofilm producer, eight (61.5%) were moderate biofilm producer and one isolate (7.6%) was strong biofilm producer. There was no weak biofilm forming Pseudomonas isolates, while 8 (62%) and 5 (38%) of the 13 were moderate and strong biofilm producers, respectively. Weak, moderate and strong biofilm formation was observed in 3 (23%), 6 (46%) and 4 *Enterobacter* isolates, respectively. Finally, weak, moderate and strong biofilm formation was observed in 2 (12%), 5 (38%) and 6 (46%) *Acinetobacter* isolates, respectively.

Since the main aim of this study was to evaluate the impact of benzalkonium chloride on biofilm of bacteria, the moderate and strong biofilm forming isolates were used for determination of MIC, and weak biofilm forming isolates were excluded. MIC of benzalkonium chloride for *P. aeruginosa, S. aureus, Enterobacter* and *Acinetobacter* was 0.14, 0.2, 0.18 and 0.17 g/ml, respectively. The effect of the disinfectant on the planktonic growth is summarized in Table 1. As shown, no turbidity was observed in the broth medium at concentrations of 2MIC, MIC and ½ MIC. The results of comparing the mean planktonic growth rate and biofilm formation of the bacteria tested exposed to different concentrations of benzalkonium chloride using Duncan's test are presented in Figures 1 and 2. In all four bacteria, absorbance increases as MIC decreases, which is due to decrease in MIC of benzalconium chloride and the subsequent increased growth of bacteria. Tables 2 and 3 show increase (%) in OD at different concentrations of benzalkonium for each of the 10 isolates during planktonic and biofilm growth stages. In each isolate, absorbance increases by reducing the concentration of the disinfectant. However, this increase in absorbance is higher in the biofilm growth stage than in the planktonic growth stage. In the biofilm growth stage, increase in absorbance by *P. aeruginosa* and *S. aureus* isolates were higher compared to *Enterobacter* and *Acinetobacter* isolates. The difference in absorbance of the planktonic growth stage of *P. aeruginosa* isolates was much more than that in other bacteria.

Table 1- Planktonic growth in the presence of benzalkonium chloride

Bacteria	2 MIC	MIC	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC
P. aeruginosa	**No growth**	**No growth**	**No growth**	**6**	**4**	**-**
S. aureus	**No growth**	**No growth**	**No growth**	**8**	**2**	**-**
Enterobacter	**No growth**	**No growth**	**No growth**	**8**	**2**	**-**
Acinetobacter	**No growth**	**No growth**	**No growth**	**7**	**3**	**-**

*** Numbers indicate the number of isolates grown**

Figure 1- Mean of planktonic growth of bacteria in the presence of benzalkonium chloride

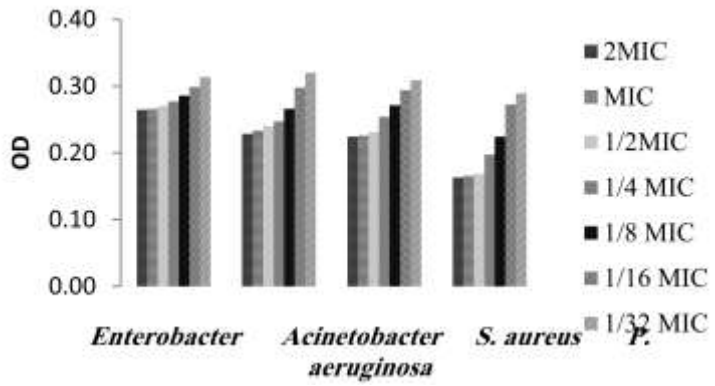

Table 2- Percentage increase in OD at different concentrations of benzalkonium chloride compared to the MIC concentrations in the biofilm growth stage

Bacterium	Initial concentration	Bacterial isolates									
		1	2	3	4	5	6	7	8	9	10
Enterobacter	MIC : 1/2	11.09	15.48	11.94	17.26	19.38	18.65	11.32	6.12	13.44	7.45
	MIC : 1/4	15.35	31.85	27.65	18.55	24.03	27.01	33.19	23.27	37.46	22.12
	MIC : 1/8	21.02	36.04	34.76	22.44	25.43	32.11	35.86	24.98	59.76	30.15
	MIC : 1/16	25.32	40.97	40.06	24.3	28.05	33.13	54.53	28.45	62.29	37.21
	MIC : 1/32	26.93	45	41.36	25.09	29.56	36.51	60.66	30.15	66.21	41.79
Acinetobacter	MIC : 1/2	19.75	19.02	17.86	17.54	10.21	30.97	23.68	5.44	24.16	35.02
	MIC : 1/4	29.32	26.11	23.56	20.1	14.95	40.9	30.54	15.4	38.43	40.9
	MIC : 1/8	34.42	33.64	30.16	23.01	16.41	45.94	40.72	20	50	48.76
	MIC : 1/16	36.39	38.56	39.62	25.29	23.17	51.3	45.96	27.48	60.32	54.83
	MIC : 1/32	40.08	42.07	49.92	26.79	28.32	57.92	51.37	36.94	64.02	59.57
S. aureus	MIC : 1/2	43.21	21.41	11.96	22.01	12.44	5.21	23.31	5.57	17.1	16.96
	MIC : 1/4	48.54	44.13	35.9	56.1	29.1	46.89	49.89	55.42	40.77	41.92
	MIC : 1/8	81.29	59.58	71.13	67.74	68.68	69.43	72.42	58.31	62.46	79.89
	MIC : 1/16	82.46	62.26	76.12	74.72	72.42	70.64	75.64	61.28	66.34	80.57
	MIC : 1/32	84.31	66.8	77.32	75.1	75.44	72.59	76.94	65.86	66.29	80.68
P. aeruginosa	MIC : 1/2	21.79	21.46	16.9	21.84	14.94	5.49	20.88	3.77	20.55	18.6
	MIC : 1/4	48.73	42.39	36.65	56.18	28.85	41.68	48.96	54.79	41.93	42.86
	MIC : 1/8	81.42	54.33	71.59	67.27	68.47	69.25	71.93	57.86	63.64	80.06
	MIC : 1/16	82.58	60.38	76.54	74.82	72.27	70.04	75.17	60.72	67.53	80.56
	MIC : 1/32	84.43	65.35	77.7	82.5	75.26	72.44	76.59	64.97	68.39	80.65

Figure 2- Mean of biofilm growth of bacteria in the presence of benzalkonium chloride

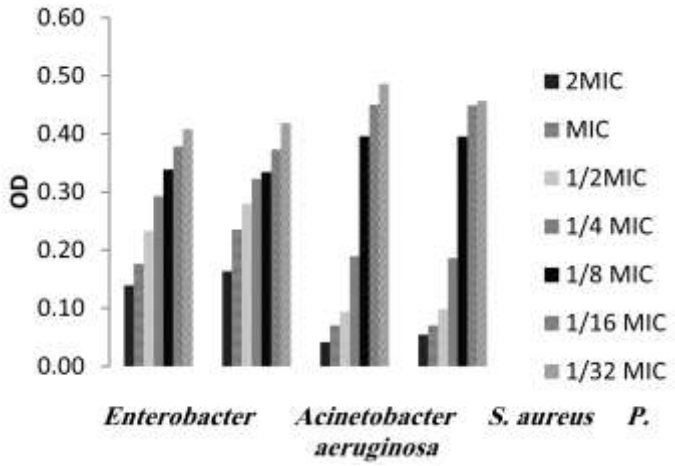

Table 3- Percentage increase in OD at different concentrations of benzalkonium chloride compared to the MIC concentration in the planktonic growth stage

	concentration	1	2	3	4	5	6	7	8	9	10
Enterobacter	MIC : 1/2	0.84	0.9	0.62	0.47	0.23	0.73	0	1.02	0.48	0.94
	MIC : 1/4	1.75	0.9	0.48	1.39	4.68	2.63	2.64	2.09	0.62	0.96
	MIC : 1/8	5.62	3.18	3.09	0.79	5.15	5.84	2.45	4.4	2.67	2.73
	MIC : 1/16	8.74	6.18	6.97	4.62	6.14	7.79	3.91	6.78	3.84	3.25
	MIC : 1/32	12.74	8.83	7.74	9.92	10.93	9.44	7.02	8.83	1.89	7.91
Acinetobacter	MIC : 1/2	0.11	0.21	0.68	1.26	1.99	0.09	0.67	6.75	1.91	1.54
	MIC : 1/4	17.1	2.09	3.08	0.44	6.36	9.22	6.14	7,94	4.2	5.11
	MIC : 1/8	10.1	5.25	5.44	7.67	11.05	13.76	10.04	11.31	6.88	7.04
	MIC : 1/16	6.06	8.52	19.17	10.36	13.04	20.33	21.89	12.78	10.07	14.38
	MIC : 1/32	0.45	10.1	20.82	8.45	22.09	24.73	24.37	20.71	13.24	15.96
S. aureus	MIC : 1/2	0	2.46	5.41	5.65	0.69	1.93	1.39	1.87	1.21	4,29
	MIC : 1/4	2.39	4.82	9.3	4	5.79	13.37	2.17	11.16	4.61	4.23
	MIC : 1/8	6.84	6.013	11.97	5.36	9.82	14.77	3.23	16.15	12	8.48
	MIC : 1/16	10.84	7.87	13.55	9.86	13.81	16.52	13.29	19.78	17.65	11.05
	MIC : 1/32	13.05	11.71	16.54	11.67	15.89	18.58	16.64	20.84	17.5	16.62
P. aeruginosa	MIC : 1/2	2.42	8	1.29	1.64	0.4	3.05	0.94	1.46	1.03	1.77
	MIC : 1/4	3.78	23.76	11.52	8.66	2.54	22.82	3.02	9.58	5.33	15.59
	MIC : 1/8	7.65	38.94	16.06	12.47	9.05	40	15.62	11.09	15.44	13.82
	MIC : 1/16	9.47	45.67	16.87	23.57	12.81	77.27	21.15	15.42	16.76	15.85
	MIC : 1/32	18.82	64.93	25.83	32.28	14.92	58.81	23.8	18.15	19.28	25.8

DISCUSSION

Controlling nosocomial infections is one of the main concerns in the medical setting since nosocomial infections are one of the common causes of mortality, increased length of hospitalization, increased hospital costs and health problems (10). Among the four bacteria studied in this study, *P. aeruginosa* and *Acinetobacter* are causes of nosocomial infections, especially in intensive care unit (11), and *Enterobacter* is the second most common cause of urinary tract infections, accounting for 10% of nosocomial infections (12). Moreover, biofilm formation by *S. aureus* in veterinary medicine and medicine is considered a chronicity factor for diseases caused by these bacteria (13). In this study, all *S. aureus, P. aeruginosa, Acinetobacter* and *Enterobacter* isolates were able to form biofilm but their biofilm formation ability was different. *P. aeruginosa* was the most powerful biofilm forming bacterium with eight moderate and five strong biofilm forming isolates. Although *S. aureus* is considered as the most important biofilm-forming bacteria in hospitals, only one of the 13 isolates of this bacterium was a strong biofilm producer. Several experimental and laboratory studies have been performed for controlling biofilm formation by bacteria. Manufacturing medical supplies and equipment made of glass and stainless steel has prevented biofilm formation to some extent (14). In laboratory studies, several compounds and chemicals such as N-

acetylcytosine, antibiotics (15) and quorum-sensing inhibitors (16) have been used to eliminate biofilms. In the present study, the impact of benzalkonium chloride on the moderate and strong biofilm forming isolates of the four tested bacteria was investigated. *S. aureus* with the highest MIC (0.2 g/ml) was found as the most-resistant bacterium against the antiseptic effects of benzalkonium chloride. This could be due to transfer of the plasmid responsible for resistance to antimicrobial agents, which is common among gram-positive bacteria. Study of Sekiguchi et al. in 2004 assessed the effect of benzalkonium chloride on *S. aureus* in hospitals of Tokyo, and reported the MIC of this disinfectant as 25.6 mg/ml (17). The MICs of this disinfectant for other three bacteria were higher than the values reported by other studies. For instance, in study of Hourai et al. (2007), the MIC of benzalkonium chloride was 0.025 g/ml for *P. aeruginosa*, which is much lower than the MIC (0.14 g/ml) reported in our study (18). The difference in the MIC values could be justified with several reasons such as difference in sampling locations. Since all the strains have been isolated from contaminated medical environments, these isolates have obtained a moderate resistance to disinfectants used in these environments. In fact, encounter of bacteria with antibacterial agents of this kind help them obtain a gradual resistance to these agents.

The effect of benzalkonium chloride on the planktonic growth and biofilm formation was evaluated using a spectrophotometer at wavelength of 630 nm. When evaluating the impact of the disinfectant.

CONCLUSION

According to the results, benzalconium chloride is efficient for control and elimination of the planktonic and biofilm forms of bacteria causing nosocomial infections. As long as cationic disinfectants are used in appropriate concentrations, they could be useful for inhibition of growth and development of biofilm formed by bacteria causing nosocomial infections. However, sub-MIC doses of benzalkonium chloride can stimulate biofilm formation. This phenomenon could have harmful effects since it is thought that biofilm formation plays an important role in transmission of nosocomial infections.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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